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2-Deoxy-D-glucose inhibition of herpes simplex virus type-1 receptor expression

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Summary

Growth of HEp-2 cells in 2-deoxy-D-glucose (2-DOG) supplemented media decreased the cells' binding capacity for herpes simplex virus type-1 KOS (HSV-1) but not vesicular stomatitis virus. HEp-2 cells tolerated up to 30 mM 2-DOG, but 2-DOG was toxic for Vero cells over 2 mM. The reduction in binding was maintained for at least 24 h even after careful removal of the inhibitor and growth in normal media. Complete regeneration of the receptor sites on HEp-2 cells was observed 8 h after mild trypsinization of cells grown in either normal or the 2-DOG supplemented media. Specific glycoprotein characteristics of the HSV-1 binding site were indicated by its inactivation upon trypsinization (0.1 mg per 5×10^5 cells for 30 s) and blocking by wheat germ agglutinin but not limulin. These results suggest that 2-DOG inhibits the proper expression of cell surface glycoprotein HSV-1 receptor sites on HEp-2 cells.

2-deoxy-D-glucose; HSV-1; receptor expression; HEp-2 cells

Introduction

2-Deoxy-D-glucose is an inhibitor of herpes simplex virus (HSV) replication and has been shown effective in reducing the severity and duration of herpetic keratitis in the rabbit model as well as human genital herpes [2]. The compound can inhibit glycosylation of cell surface proteins and lipids and alter their proper expression on the cell membrane [2,10,14,19]. Incorporation of 2-DOG truncates the high mannose intermediate form of the glycoprotein, thus altering its structure, expression on the cell surface

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[19], and potentially, its activity. Glycosylation of the HSV-1 glycoproteins is also inhibited by 2-DOG [3]. This prevents proper interaction of the virus with the cell as indicated by a reduction in virus penetration, infectivity [18] and syncytia formation [6]. The interaction of HSV with the target cell might also be inhibited if a glycosylated cell surface receptor were altered.

The receptor for HSV is expressed on many different cell types [17]; however, specific receptor sites are required for HSV binding. Preliminary characterization of the HSV-1 receptor has shown that virus binding to GMK-AH1 and BHK cells [1,11] is lost following trypsinization of the cells and for sensory neurons, can be blocked by the lectin, wheat germ agglutinin (WGA) but not limulin [25]. These results indicate that a glycosylated protein is important for HSV-1 binding. However, several studies indicate that the characteristics of the HSV receptor site may differ for different cells [23,24].

In this report we show that the interaction of HSV-1 with HEp-2 cells is altered following growth of the cells in 2-DOG. In addition, HSV-1 binding sites on HEp-2 cells resemble the HSV-1 binding sites on GMK-AH1 [11] and neuroblastoma [25] cells with respect to trypsin sensitivity and WGA blocking of attachment. Our studies suggest that HSV-1 binds to glycoprotein receptor sites on HEp-2 cells which contain *N*-acetylglucosamine and/or sialic acid residues; that 2-DOG, an inhibitor of glycosylation, inhibits the proper cell surface expression of HSV-1 receptor sites and that these receptor sites turn over very slowly in non-growing cells.

Materials and Methods

Reagents and radioisotopes

2-Deoxy-D-glucose (2-DOG) grade III, and wheat germ agglutinin (WGA) were from Sigma Chemical Company, St. Louis, MO. *Limulus polyphemus* agglutinin (limulin) was kindly provided by Dr. J.M. Kehoe, Northeastern Ohio Universities College of Medicine. [*methyl*-^{3}H]Thymidine was from ICN Pharmaceuticals, Irvine, CA. Other chemicals were from Fisher Scientific, Pittsburgh, PA. Phosphate-buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4. Tris-buffered saline (TBS) consisted of 25 mM Tris-HCl, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.7 mM NaH_2PO_4, pH 7.4. TNE buffer consisted of 10 mM Tris-HCl, 100 mM NaCl and 1 mM disodium EDTA, pH 7.4.

Cells and virus

HEp-2 and Vero cells were grown at 37°C in 75-cm^2 flasks (Corning Glassworks, NY) using Eagle's modified minimal essential medium (Auto Pow, Flow Laboratories, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (K.C. Biologicals, Kansas City, MO), gentamycin (50 μg/ml), L-glutamine (0.03%) and non-essential amino acids (EMEM). Maintenance medium was prepared as described above, with 2% newborn calf serum instead of FBS.

HSV-1 (KOS strain) stocks were prepared by infection of HEp-2 cells at a multiplici-

ty of infection (MOI) of 0.01–0.05. Infectious virus titers were assayed by plaque assay on Vero cells with maintenance medium containing methyl cellulose (2%) as an overlay [5]. VSV (Indiana strain) was obtained from Dr. R.R. Wagner, and was propagated in human foreskin fibroblasts (HFF) (M.A. Bioproducts, Walkersville, MD).

Radiolabeling of HSV-1

Confluent monolayers of HFF cells in 60-mm petri dishes (Corning Glassworks, NY) were infected with HSV-1 at a MOI of 1 at 37°C for 1 h and then incubated at 34°C in maintenance media. At 4 h postinfection, 50 μl of [*methyl*-^{3}H]thymidine (1 mCi/ml) was added to the medium, effectively labeling only viral DNA. After 2–3 days, extracellular virus was centrifuged at 27 000 × *g* for 1 h at 4°C. The resulting viral pellet was then resuspended in 1 ml of TBS and centrifuged through a discontinuous sucrose density gradient (10–60%) in TNE buffer at 120 000 × *g* for 1 h. The peak fractions containing both radioactivity and infectivity were pooled and used for these studies.

Adsorption of HSV-1 to trypsinized cells

HEp-2 cells grown in 24-well tissue culture plates (2×10^5 cells/well) (Corning Glassworks, NY) were washed three times with PBS and incubated with PBS or trypsin (0.1 mg/ml) in PBS at room temperature. Proteolysis was stopped by washing the cells three times with EMEM at different times with respect to the addition of trypsin. The fetal bovine serum (10%) in the medium was used to stop the trypsin activity. The plate was then cooled to 4°C and an aliquot (400 μl) of HSV-1 in cold EMEM was added to each well. HSV binding but not penetration occurs at 4°C [9]. The virus was incubated with the cells for 3 h at 4°C in maintenance media with constant shaking. The cells in the plate were then washed 3 times to remove unbound virus and incubated at 37°C in 400 μl of EMEM for 1 h to allow uptake of bound virus. The extent of virus binding in each well was then determined by plaque assay [5].

The kinetics of HSV binding was determined by adding identical aliquots of [^{3}H]thymidine labeled HSV-1 in PBS to each of the trypsin or mock treated HEp-2 cell monolayers at 4°C. Following different incubation periods, unbound virus was removed by washing and the monolayer was solubilized in 10% SDS and assayed for radioactivity.

Effect of cell bound lectin on HSV-1 attachment

HEp-2 cells grown in 24-well tissue culture plates were washed three times with PBS and incubated at 37°C for 30 min with either PBS plus 1 mM $CaCl_2$ or different concentrations of WGA or limulin in PBS plus 1 mM $CaCl_2$ (200 μl per well). Unbound lectin was removed by extensive washing of the cells. The cells were then cooled to 4°C and HSV-1 binding and infection were determined as described for trypsinized cells.

Virus binding to cells grown with 2-DOG

HEp-2 cells were passaged and grown in EMEM or EMEM supplemented with varying concentrations of 2-DOG in 24-well tissue culture plates. After approximately 3–4 days, confluent monolayers of cells were washed extensively with TBS and infected

at 37°C for 1 h with identical aliquots of HSV-1 (MOI <0.01). Unbound virus was removed by washing and the number of plaques was determined 3–4 days later. VSV binding and infection of HEp-2 cells grown with and without 10 mM 2-DOG was determined using the same protocol.

Assay of HSV-1 penetration into HEp-2 cells

The kinetics of HSV-1 penetration was analyzed by protection of the virus from acid inactivation with respect to time following a temperature shift from 4 to 37°C [15]. Confluent cell monolayers in 24-well plates were washed extensively to remove 2-DOG and then incubated for 4 h with the virus in PBS at 4°C. This temperature was chosen to limit virus penetration yet allow virus binding to the cells. The inoculum was adjusted to yield 30–200 plaques per monolayer. Unbound virus was washed away and the cells were shifted to 37°C to allow the virus to penetrate into the cells. After different time intervals, 1 ml of dilute HCl in saline (pH 3.0) was added for 1 min to destroy extracellular virus. The cells were then washed 3 times with maintenance medium and prepared for plaque assay.

Kinetics of HSV-1 receptor regeneration following trypsin or 2-DOG treatment

HEp-2 cells grown to confluency in a 24-well tissue culture plate with normal or 10 mM 2-DOG supplemented medium for 4 days were washed three times with PBS and incubated with PBS alone or trypsin in PBS (0.1 mg/ml, 200 µl per 5×10^5 cells) at room temperature. After 2 min, the cells were washed 4 times with EMEM to stop the trypsin action and then incubated at 37°C in 300 µl of EMEM. At different times following trypsinization, identical aliquots of HSV-1 were added to the cells and incubated for 1 h at 37°C. Unbound virus was removed and the bound virus was determined by plaque assay.

Results

HSV-1 infection of cells grown with 2-DOG

The effects of 2-DOG supplementation of media on HEp-2 cell growth are shown in Fig. 1. Different concentrations of 2-DOG were incorporated into the normal EMEM growth medium immediately following passage of the cells. The EMEM contains 6 mM glucose. Following an initial crisis resulting in the loss of approximately 60% of the cells, the rates of growth of the cell culture returned to that of the control for cells grown with 2–10 mM 2-DOG. Higher concentrations of 2-DOG inhibited growth of the cells. However, growth with 20 and 30 mM 2-DOG did not prevent the formation of confluent monolayers. In contrast, supplementation of the EMEM with greater than 2 mM 2-DOG was toxic to Vero cells causing rounding of cells and loss of the monolayer within 24 h. Most of the subsequent experiments were performed following 3 days' growth of HEp-2 cells in 10 mM 2-DOG. Toxicity was minimized at this time and concentration and the cell monolayers most resembled that of the controls with respect to growth rate and appearance.

HSV-1 infection of confluent monolayers of cells grown with varying concentra-

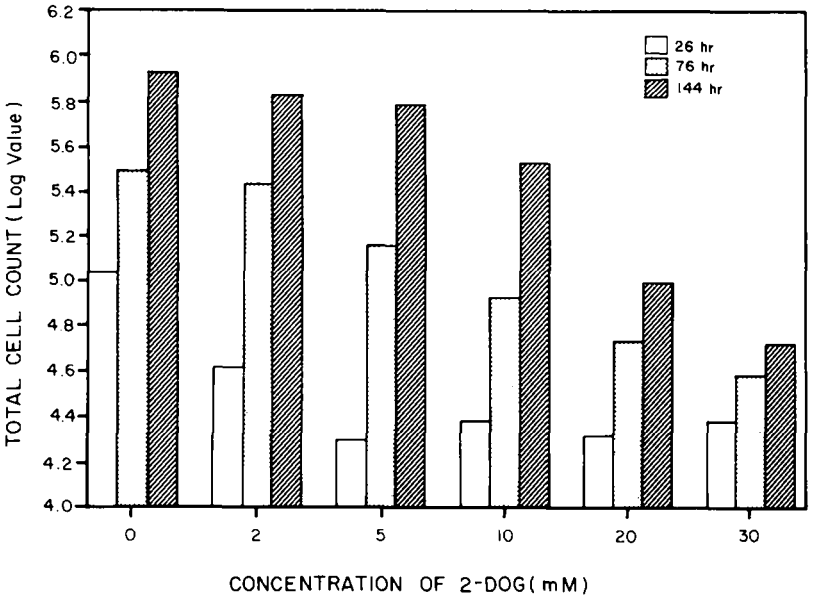
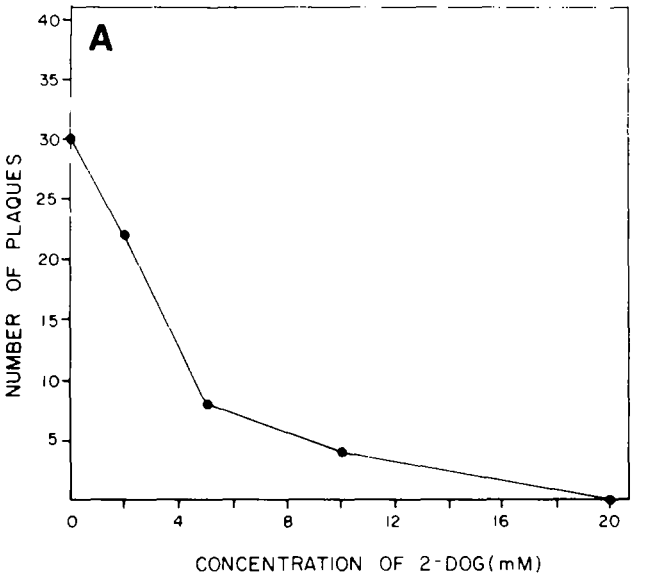


Fig. 1. Effect of 2-DOG on the growth of HEp-2 cells. Growth of cells in normal media supplemented with different concentrations of 2-DOG was determined by viable cell count (± 0.1 log units) following trypsinization of monolayers grown in a 24-well tissue culture plate. Each well was initially seeded with 8×10^4 cells (log value = 4.9).

tions of 2-DOG was tested following removal of the compound from the media. As shown in a representative experiment (Fig. 2A) HEp-2 cells grown with increasing concentrations of 2-DOG and infected with equal amounts of HSV-1 for 1 h at 37°C produced fewer viral plaques. Approximately a 60–80% reduction in virus infection was observed for HEp-2 cells grown with 10 mM 2-DOG. No virus infection was observed under these conditions in cells grown with 20 mM 2-DOG. This method was also used to study the kinetics of binding of HSV-1 (Fig. 2B). As in the previous experiment, a subsaturating amount of virus was used to allow plaque assay of the bound virus. The rate of virus binding to the cells grown with 10 mM 2-DOG was slower than for control cells, however, with longer incubation periods, both cell cultures sustained similar infectivity. The rate of binding of [^{3}H]thymidine labeled HSV-1 to HEp-2 cells grown with 10 mM 2-DOG was at least 10-fold slower than control cells (Fig. 3). These are indications of depletion or modification of the virus binding sites.

The kinetics of HSV-1 penetration into HEp-2 cells grown in 2-DOG was assayed by protection from acid inactivation of extracellular virus. Prior to assay, cells grown for 3 days with 10 mM 2-DOG or control cells were washed extensively and incubated with virus for 4 h at 4°C to maximize virus attachment and allow synchronization of virus penetration upon a temperature shift to 37°C. A 10-fold larger inoculum of HSV was required in these experiments for the 2-DOG grown cells. A 5–7-min lag prior to internalization is routinely observed. This is followed by a rapid uptake of virus, which



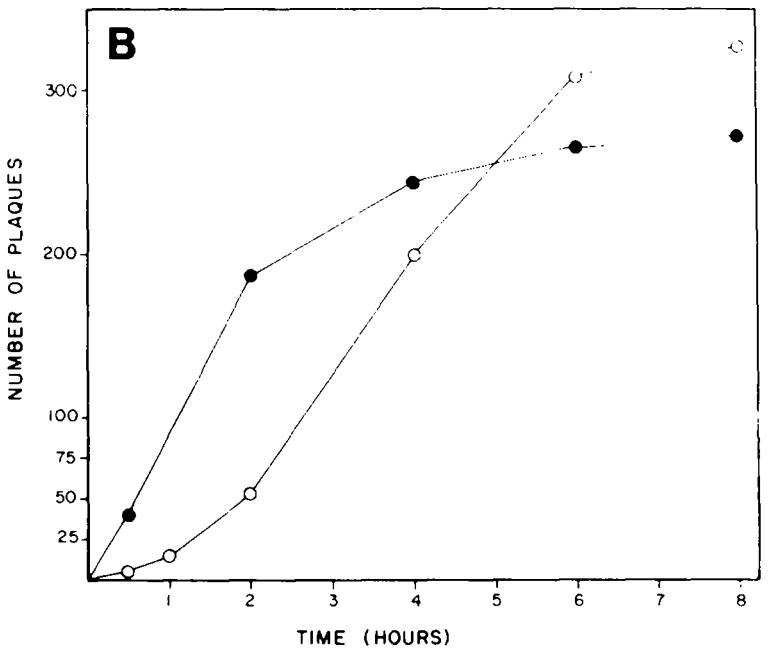


Fig. 2. HSV-1 infectivity of HEp-2 cells grown with 2-DOG. (A) Cells were grown with varying concentrations of 2-DOG for 3 days, the 2-DOG was washed away and the cells incubated with HSV-1 at 37°C for 1 h in normal EMEM plus 10% FBS. Unbound virus was removed and the cells prepared for plaque assay. (B) Replicate monolayers grown in 10 mM 2-DOG, as above (○——○) or normal medium (●——●) were incubated with equivalent concentrations of virus in PBS for varying times at 37°C. Unbound virus was washed away and the cells prepared for plaque assay.

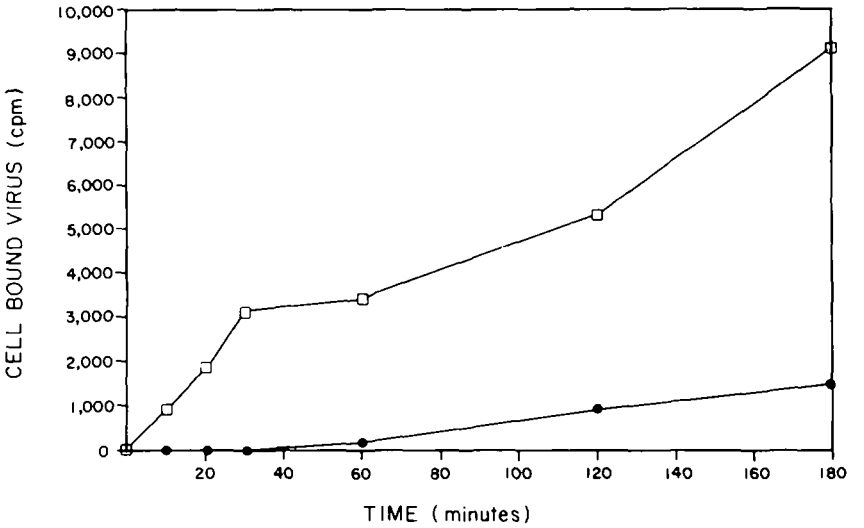


Fig. 3. Binding of [^{3}H]thymidine labeled HSV-1 to HEp-2 cells grown in 10 mM 2-DOG supplemented (●——●) or normal (□——□) medium. Cells were prepared and assayed as described for Fig. 2.

is complete within 30 min [15]. Although fewer infectious virus particles bound to the cells grown with 2-DOG than control cells, the rates of internalization of the cell bound HSV-1 into cells grown with 10 mM 2-DOG and cells grown in the absence of 2-DOG were similar (Fig. 4).

VSV infection of cells grown with 2-DOG

The effect of growth of HEp-2 cells in 10 mM 2-DOG on VSV (10 to 400000 PFU/monolayer) infection and replication was studied in order to determine the capacity of the treated cells to support a virus infection. Recent reports suggest that VSV does not require a glycoprotein receptor for infection [15]. No difference in the number of plaques was observed for untreated versus treated cells at any MOI tested.

Binding of HSV-1 to trypsinized cells

The protein nature of the HSV-1 receptor sites on HEp-2 cells was tested by sensitivity to trypsinization. Monolayers of the cells were subjected to mild trypsinization conditions. Trypsin treatment under these conditions for up to 3 min did not disrupt the cell monolayer. HSV-1 binding to trypsin or mock treated cells was assayed following a 3-h incubation with virus at 4°C, removal of unbound virus and incubation at 37°C. As can be seen in Fig. 5, HSV-1 binding to HEp-2 cells was very sensitive to trypsin treatment. A greater than 90% reduction in plaques was observed with only a 30-s trypsin treatment of HEp-2 cells. Vero cell binding of HSV-1 was more resistant to trypsin treatment and required at least 3 min to show a similar effect.

The kinetics of virus binding to the trypsinized cells was examined with [^{3}H]thymidine labeled HSV-1 at 4°C as a further indication of the number of effective receptor sites available on the cell surface [10]. As can be seen in Fig. 6, only low level or

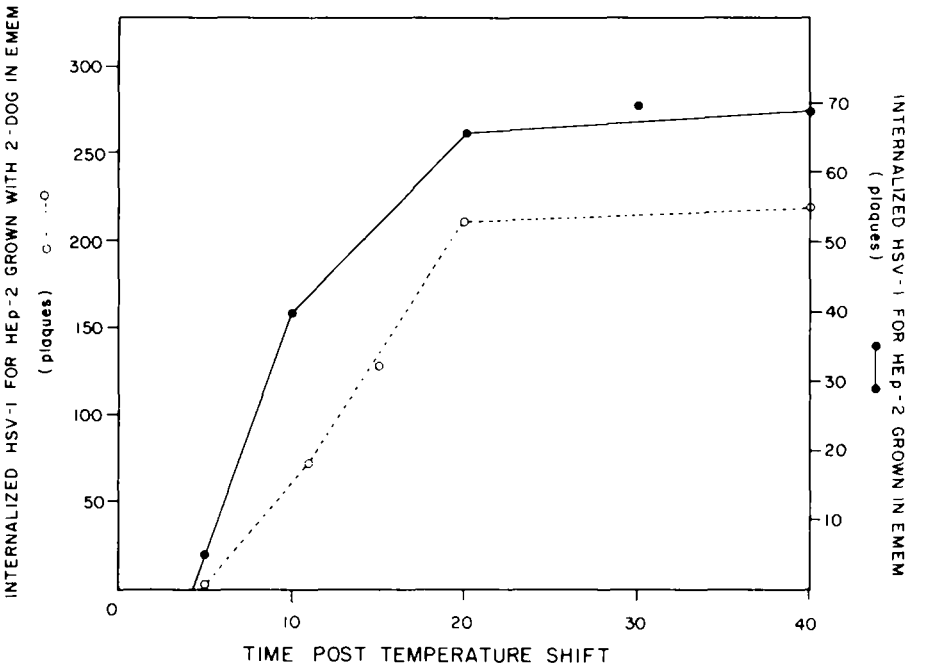


Fig. 4. Kinetics of HSV penetration. HEp-2 cells grown in separate 24-well plates with 10 mM 2-DOG supplemented EMEM for 3 days (o---o) or normal EMEM (●——●) were incubated with HSV-1 for 4 h at 4°C. The inoculum size for the 2-DOG grown cells was approximately 10 times that for the normal grown cells. The unbound virus was washed away and the cells rapidly shifted to 37°C. At different times post temperature shift, the cells were treated with pH 3.0 saline for 1 min, washed with medium and then overlayed with methylcellulose containing medium for plaque assay. Virus penetration is indicated by the survival of infectious virus.

non-specific binding of the virus to the trypsinized cells was observed within a 2-h period.

Lectin blocking of HSV-1 attachment to cells

The carbohydrate specificity of HSV-1 binding was indicated by the ability of WGA but not limulin to block HSV-1 binding. HEp-2 cells were incubated with sublethal concentrations of lectin (less than 500 μg per well per 5×10^5 cells) for 30 min and unbound lectin was washed away prior to the addition of virus. As can be seen in Fig. 7, HSV-1 binding and infection of HEp-2 cells decreased with increasing amounts of added WGA. Maximal blocking of HSV-1 binding sites was observed at approximately 100 μg WGA with 90% inhibition at 10 μg. Binding of ^{3}H-labeled HSV was similarly inhibited by WGA (data not shown). WGA specifically binds *N*-acetylglucosamine but also interacts with sialic acid [7]. Limulin, another sialic acid binding lectin, had no activity on HSV-1 binding to HEp-2 cells. These results indicate that specific glycoconjugates containing *N*-acetylglucosamine and/or multiply substituted terminal sialic acid groups on HEp-2 are important for the binding of HSV-1.

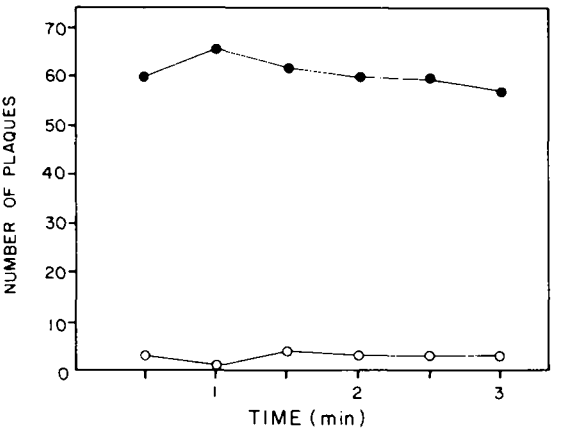


Fig. 5. Effect of mild trypsinization of HEp-2 cells on HSV-1 binding at 4°C. Cell monolayers grown in 24-well tissue culture plates were either treated with 200 µl of 0.1 mg trypsin/ml per well (o——o) or PBS (●——●) for varying periods of time.The trypsin was washed away with EMEM +10% FBS and the cells incubated at 4°C with virus for 3 h. Unbound virus was removed and the cells overlayed with maintenance medium containing 2% methylcellulose for plaque assay. Trypsinization conditions were insufficient to remove cells from the monolayer.

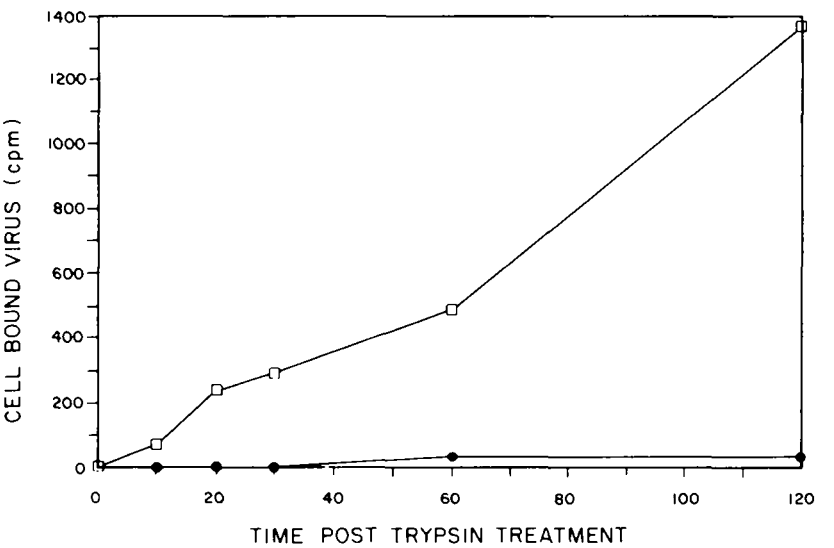


Fig. 6. Kinetics of [^{3}H]thymidine labeled HSV-1 binding to HEp-2 cells. Cells were either trypsin (●——●) or mock (□——□) treated as described for Fig. 5. After removal and serum diminution of residual trypsin activity, identical aliquots of ^{3}H-labeled HSV-1 were added and incubated with the cells for varying periods of time at 4°C. The cells were then washed, solubilized in SDS and assayed for radioactivity.

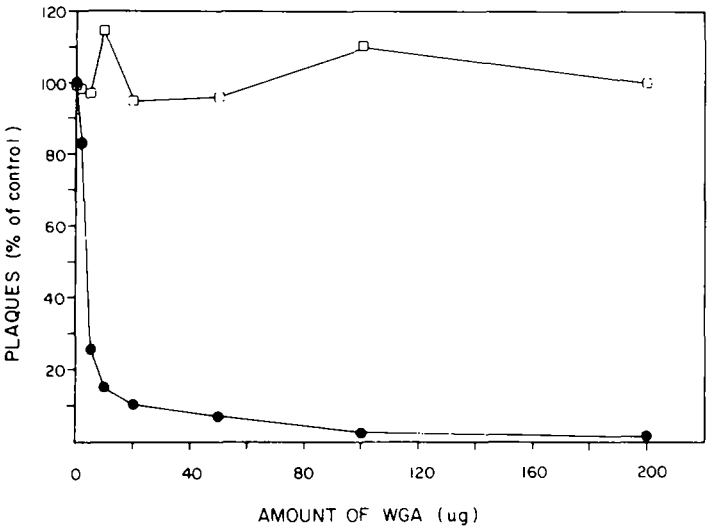


Fig. 7. Lectin competition for HSV-1 binding. Cells were incubated with zero (control), varying amounts of WGA or limulin for 30 min at 37°C, washed free of unbound lectin and cooled to 4°C prior to addition of virus. HSV-1 binding was measured by the number of plaque forming virus bound to the cells after a 4-h period. WGA with HEp-2 cells (●——●); limulin with HEp-2 cells (□——□).

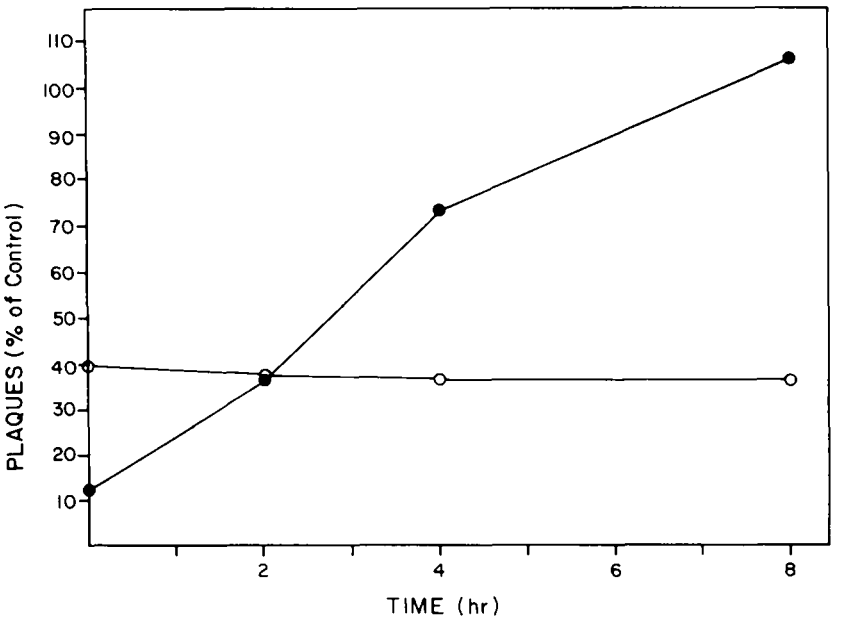


Fig. 8. Kinetics of regeneration of HSV-1 receptors of HEp-2 cells following trypsinization (●——●) or growth with 2-DOG (○——○). Cells were grown with 10 mM 2-DOG for 3 days or treated with 0.1 mg/ml trypsin for 3 min. The trypsin and 2-DOG were removed as described for Figs. 1 and 5 and the cells were incubated in normal medium for varying periods of time at 37°C. HSV-1 was then added for 1 h at 37°C, unbound virus washed away and bound virus detected by plaque assay. Number of plaques produced by mock treated, infected cells represent the control.

TABLE 1

Regeneration of HSV-1 receptors on HEp-2 cells grown with 2-DOG

Cellular conditions	Number of plaques (± 10%)[c]
Normal cells[a]	179
Cells grown with 10 mM 2-DOG[a]	39
Cells grown with 10 mM 2-DOG, trypsinized and regrown in normal media[b]	170

[a] Confluent monolayers of HEp-2 cells were either grown in EMEM +10% FBS or EMEM +10% FBS supplemented with 10 mM 2-DOG for 3 days. The 2-DOG was removed and the cells incubated with fresh EMEM for 24 h prior to assay.

[b] Cells grown with 2-DOG as above were removed from the monolayer with trypsin, the cells centrifuged, washed with EMEM +10% FBS to remove the trypsin and replated. The cells were allowed to attach and grow for 24 h.

[c] Identical aliquots of HSV-1 were added to each monolayer, the cells incubated for 4 h at 4°C, washed to remove unbound virus and shifted to 37°C to allow plaque formation as described in Methods.

Regeneration of the HSV-1 receptor sites on HEp-2 cells following trypsin or 2-DOG treatment

As can be seen in Fig. 8, HSV-1 binding and infection of mildly trypsinized cells returned to control levels within 8 h of the initital proteolysis. Unlike the trypsin treated cells, confluent monolayers of HEp-2 cells grown with 10 mM 2-DOG for 3 days failed to regenerate HSV-1 binding capacity within 8 h and for up to 24 h after careful removal of the compound. However, when these cells were trypsinized, replated in EMEM and maintained at 37°C for 24 h, complete recovery of control levels of HSV-1 binding capacity was observed (Table 1). This indicates that the 2-DOG effect on the HSV-1 binding capacity of HEp-2 cells can be reversed but that it requires growth stimulation of the monolayer by a treatment such as trypsinization. It is also further indication that the cells grown with 2-DOG remain viable.

Discussion

In this study we show that growth of HEp-2 cells in media supplemented with 2-DOG decreases their infectability by HSV-1. This decreased capacity for infection could have been due to either inhibition of the initial virus-cell interaction, toxicity of 2-DOG to the target cells or inhibition of proper virion synthesis. The latter two possibilities are unlikely since our experiments were performed on 2-DOG treated cells with growth rates and appearance similar to control cells and capable of replicating another virus, VSV. HEp-2 cells grown with 10 mM 2-DOG for 3 days formed confluent monolayers of viable cells which reverted to control cell characteristics following removal of 2-DOG and trypsinization of the cells. The HEp-2 cells tolerated a supplementation of up to 30 mM 2-DOG in normal, glucose containing media. However, 2-DOG was toxic to Vero cells. Selective toxicity of 2-DOG has been observed for other cells [13,20] and is based on the cell's ability to phosphorylate

2-DOG [2]. In all experiments, the 2-DOG containing media were replaced with normal glucose-containing media prior to virus addition in order to limit any effect of 2-DOG on glycolysis or viral macromolecular synthesis. The viability of the cells and their capacity to support virus infection was also indicated by the permissiveness of the 2-DOG treated cells to VSV. No difference in infectability could be observed between the normal and 2-DOG treated cells. VSV was studied because it does not necessarily require a glycoprotein receptor [16] and hence its replication would not be affected by a 2-DOG induced alteration in glycoprotein receptor expression. Another study has shown that HeLa cells treated with up to 50 mM 2-DOG immediately prior to addition of virus are capable of binding, internalizing and synthesizing the early and late proteins of adenovirus 2 [20].

The interaction of HSV-1 with several cell types has been shown to require a glycoprotein structure sensitive to trypsin which is blocked by WGA but not limulin [25]. These are also the properties of the HSV-1 binding site on HEp-2 cells. Hence, a modification in glycoprotein synthesis or expression upon growth of cells with 2-DOG [2] would be expected to affect the interaction of HSV with its target cell. The significant decrease in the extent of infectious virus binding at 37°C and the rate of [^{3}H]thymidine labeled HSV-1 binding at 4°C following growth in 2-DOG is indication of a modification or depletion of an HSV binding site. Although the penetration of HSV into the cell is also an early step in infection which is likely to depend upon the structure of a viral receptor site, the kinetics of penetration of bound virus was the same for cells grown with 2-DOG and control. This is in contrast to results for herpes simplex virions grown in the presence of 2-DOG which are able to attach but not penetrate efficiently into the target cell [6,18].

Unexpectedly, the decrease in HSV binding capacity observed for the cells grown in 2-DOG was not reversed following removal of the compound from the media. This suggests that growth in 2-DOG alters expression of the HSV receptor and that the HSV-1 receptor sites have a very slow turnover rate in non-growing cells. Trypsinization of the cell monolayer stimulates cell growth, division and turnover of cell surface proteins [4] which apparently includes the HSV-1 binding site.

Our studies show that the growth of target cells with 2-DOG can decrease their infectability by HSV-1, even in the presence of physiological levels of glucose. This can be attributed to a decrease in virus binding capacity resulting from an alteration in the expression of glycoprotein receptor sites. Hence, 2-DOG can inhibit HSV-1 infection by preventing proper synthesis and expression of both the viral [2,6,18] and cellular structures required for the initial steps of infection.

Acknowledgements

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